PHENOTYPIC CHANGES IN DENDRITIC CELLS WHEN CHALLENGED WITH COWPOX VIRUS

By

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With the increasing threat of bioterrorism, the need to understand the pathological mechanisms behind poxvirus infections has become of paramount importance. Poxviruses are one of the most feared applications of bioterrorism, partly due to the virus' rapid dissemination in the target population, as well as its high morbidity and mortality. Poxvirus, unlike many other viruses, maintains the ability to modulate its host's immune system in a manner that promotes its survival and infectivity. The objective of this study was to determine how poxvirus interacts with the host's primary antigen recognition system, dendritic cells (DCs), a major component of the immune system that has the ability to link the innate and acquired immune responses. Immature DCs serve as sentinels in peripheral tissues where they undergo a programmed maturation to become an efficient antigen presenting cell characterized by high surface expression of MHC class II and CD86, production of high levels of IL-12, and ability to prime naïve T-cells. Little is known, however, regarding the DC maturation response to

poxvirus infection. Examination of DC surface markers as well as cytokine production demonstrated that DCs do not undergo a vigorous activation or maturation in response to cowpox virus infection. Furthermore, concomitant cowpox virus infection also attenuated CD86 expression when DCs were subsequently stimulated with bacterial lipopolysaccharide (LPS). In DCs infected with poxvirus, production of TNF-α and IL-12p70 was also significantly reduced and in some cases undetectable when compared to DCs that were stimulated with LPS alone. Interestingly, levels of IL-10 were significantly increased in the poxvirus infected DCs when compared to DCs stimulated with LPS, which had relatively low to undetectable levels. These findings suggest that DCs are not readily activated by cowpox virus infections, and more interestingly, cowpox virus infections alter the subsequent DC response to a microbial challenge. The findings confirm that poxvirus infections alter innate immune responses, and may explain the ability of poxvirus infection to evade recognition by the innate immune system.

CHAPTER 1
INTRODUCTION

Dendritic Cell Biology

Steinman and his colleagues first characterized dendritic cells (DCs) in the 1970s as a novel splenic cell population with immunological properties. Since then, DCs have become the subject of much research interest, particularly in microbial recognition [1-3]. DCs, characterized by a stellate morphology, exist in all lymphoid tissues and have been observed in many areas of the reticuloendothelial system. When compared to other antigen presenting cells (APCs) of the immune system, DCs have a 10-100 fold greater capacity to stimulate naïve T-cells than other APCs [4-7]. After encountering foreign antigen or danger signals, DCs can amplify their ability to present antigen to T-cells through various mechanisms [8,9]. Additionally, in comparison to other APCs, DCs lack the ability to eliminate antigen via antibody production or microorganism destruction [8,9]. While DCs exist as one of several phenotypically different subtypes, our studies are focusing on the bone marrow-derived $CD34^+$ DC population [9]. These myeloid DCs are capable of accessing most tissues via the bloodstream. Immature DCs are initially directed to peripheral tissues where they act as sentinels for the detection of self and nonself. Found primarily in tissues such as the skin, lung epithelium, gut and other interfaces with the environment, immature dendritic cells continuously sample their environment through endocytosis and pinocytosis. In the absence of any stimulus, immature DCs migrate from peripheral tissues to draining lymph nodes, and are relatively non-immunogenic because they express low levels of MHC class II and co-

stimulatory molecules, and exhibit little capacity to present antigen [10]. However, in the event that an immature DC encounters a foreign antigen or a danger signal in the periphery, they migrate to the draining lymph node and lose their capacity to take up antigen. The activated DC increases surface expression of MHC-peptide complexes, upregulates expression of costimulatory molecules such as CD86, and increases production of proinflammatory cytokines such as IL-12p70 and tumor necrosis factor alpha (TNF-α) [11] (Figure 1).

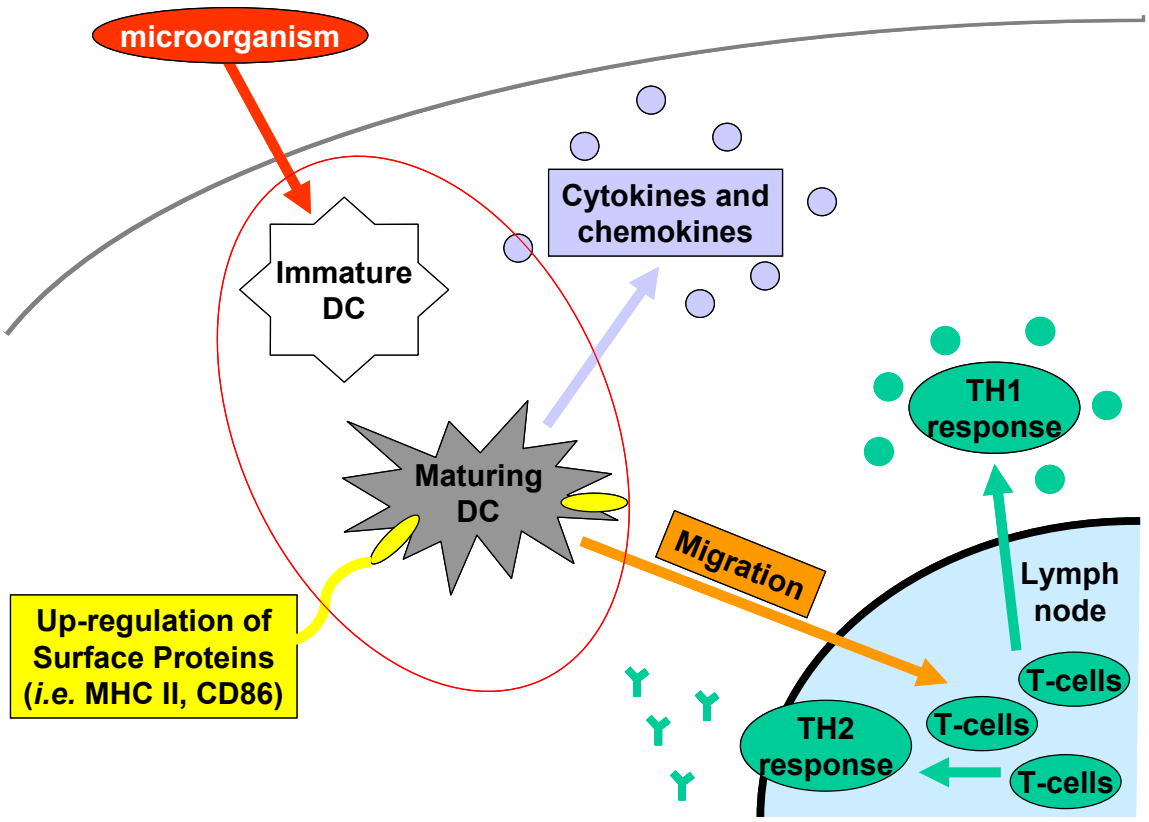

Figure 1. Schematic diagram depicting a DC response to external stimulus and subsequent activation of T-cells

Note: As demonstrated here, the immature myeloid DC rests in the periphery until it encounters stimulation, such as a foreign antigen or an endogenous 'danger signal'. Subsequently the DC undergoes a maturation process. The mature DC attains the ability to migrate to the local lymph nodes by altering its pattern of chemokine receptors, and induces either a Th1 or a Th2 response, thus linking the innate and acquired immune systems.

Maturation of DCs can be initiated by a number of different endogenous and exogenous stimuli, including proinflammatory cytokines (TNF-α), CD40 ligand, bacterial cell antigens, as well as some viral antigens. Recent interest has focused on a family of toll-like receptors, which currently number ten, that are involved in the recognition of both pathogen associated microbial products as well as endogenous danger signals [12]. Interestingly, the nature of the stimuli determines the type of DC and lymphocyte response (e.g., Th1 vs. Th2) [13,14]. For example, extracellular bacterial infections and their products appear to stimulate the maturation of a type I DC, characterized by their ability to produce high levels of IL-12 and to generate a Th1 type of acquired immune response. In contrast, helminthic infections appear to induce a more type II DC maturation response associated with elevated production of IL-10 and the generation of a Th2 type of acquired immune response. This property of DCs reaffirms that they can serve as the link between the innate and acquired immune systems. DC development is guided by environmental factors which may become the Achilles' heel for immune responses. Therefore, it is critical to fully understand this pathway and how it progresses in response to different pathogens.

Poxvirus Pathogenesis

Poxviruses, along with a number of other viruses, share mechanisms capable of modulating the host immune response. Large DNA viruses like poxviruses express a wide array of immune regulatory genes, taking up approximately 50% of the total genome, dedicated to protecting the virus from the host immune system [15]. Poxviruses contain linear double-stranded DNA genomes with termini that contain covalently closed hairpin loops. *Chordopoxvirinae*, the family of poxviruses that infects vertebrates, has a

genome that can range in size from 135,000 base pairs to 289,000 base pairs. Genes that are located toward the center of the genome are generally highly conserved among all poxviruses and tend to be involved in common viral functions such as replication and virus assembly [15,16]. Genes that are located towards the termini are typically more variable and are involved in immune response modulation specific to the individual poxvirus.

Poxviruses have developed two classes of immunoregulatory proteins: those that share and those that do not share sequence homology with host proteins [16]. These immunoregulatory proteins, including cytokine receptor antagonists and caspase inhibitors, have a dramatic effect on the classical maturation process of DCs, a phenomenon that is not well understood and is consequently the focus of this study.

Many poxviruses possess a protein that is capable of down regulating MHC class I, which is responsible for presenting all intracellular antigens. Down regulation of MHC class I would lead to decreased immune recognition by $CD8^+$ CTLs of any viral agents. The mechanism of action is thought to occur at the endoplasmic reticulum, where these immune regulatory proteins target newly formed MHC class I molecules for retention and degradation via an endosomal pathway [17,18]. In addition to proteins that regulate the cell-mediated immune response, poxviruses also employ a number of mechanisms that modulate the extracellular environment. Most of theses mechanisms utilize a soluble receptor homolog that binds free inflammatory cytokines and prevents them from binding to their receptors, ultimately decreasing the inflammatory response and inhibiting CTL-mediated cytolysis. Poxviruses also have receptor homologs to bind many different cytokines including interferon (IFN) α, β, and γ, IL-18, and the TNF family of molecules

[19-23]. Another important immune regulatory mechanism is the ability to inhibit apoptosis of the host cell. Poxviruses have a strategy that disrupts the apoptotic pathway by preventing the activation of caspase-8, which is a protein responsible for initiating apoptosis secondary to death domain activation. The most extensively studied anti-apoptotic protein is the CrmA protein of cowpox viruses [24]. CrmA acts on the different apoptotic pathways in many different manners. CrmA is a serine protease inhibitor (serpin) and exerts this action on granzyme B, which is a serine protease that is delivered to virally infected cells by NK cells and CTLs. Granzyme B is the initiator protein of perforin-dependent apoptosis [25]. CrmA also has the ability to inhibit caspase-8 and caspase-10 in order to inhibit apoptosis at other critical points in the pathway [26].

Poxvirus and Dendritic Cell Interactions

Since DCs play a pivotal role in the recognition of nonself during microbial invasion, its role in the early detection of poxvirus infection would be crucial to the development of a successful immune response. There have been a number of earlier studies that have examined the interactions between DCs and poxvirus. Most of these studies have employed vaccinia virus strains and human peripheral blood monocyte derived DCs. These studies have generally shown that replicating vaccinia virus strains will infect human derived DCs and will express early virus genes, but are often nonpermissive for viral DNA replication or late protein synthesis [27]. Furthermore, the studies suggest that poxviruses fail to activate human DCs, and these DCs become resistant to subsequent maturation by endogenous activation agents [28-30]. However, those studies have not examined whether murine bone marrow derived DCs respond in a similar fashion to cowpox infections, whether the infected DCs are still responsive to an extrinsic activation agent, like bacterial lipopolysaccharide (LPS), and whether the

resistance to activation by LPS is limited only to those DCs directly infected by cowpox virus, or whether the suppression of activation is secondary to a bystander effect.

The specific aims of the current study were therefore three-fold: 1) to determine whether poxvirus could infect murine myeloid DCs, 2) to examine whether infection with poxvirus would lead to maturation of those cells directly infected by the virus, as well as by DCs that were not directly infected (bystander effect), and 3) to determine whether poxvirus infection altered the capacity of myeloid derived DCs to undergo maturation in response to an extrinsic inflammatory challenge (bacterial LPS). In this manner, we hope to determine whether poxvirus infections activate the innate immune system in a manner comparable to that seen with other TLR receptor based ligands, and whether poxvirus infections modulate the subsequent ability of DCs to respond to subsequent inflammatory challenges. This latter point is particularly relevant to the secondary opportunistic infections that may contribute to the high mortality associated with poxvirus infections.

CHAPTER 2
MATERIALS AND METHODS

Generation of Bone Marrow Derived DCs

Female C57/BL/6 mice between eight and twelve weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Additional IL-10 null (C57BL/6$^{il10-/-}$) and TNFR I null (C57BL/6$^{tnfr1-/-}$) mice were bred locally and maintained at the Animal Care Services, University of Florida. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida College of Medicine prior to these studies. DCs derived from murine bone marrow were generated as described by Oberholzer et. al. [31]. Briefly, bone-marrow cells harvested from the femur and tibia of C57BL/6 female mice were depleted of red blood cells by lysis with ammonium chloride. Thereafter, approximately 10^6 cells were cultured on 24-well plates (Costar, Corning, NY) in RPMI 1640 (Cellgro, Herndon, VA) with 10% heat inactivated fetal calf serum, 0.000375% 2-mercaptoethanol (Sigma Chemical Co., St. Louis) and 1% penicillin-streptomycin-neomycin (Gibco, Grand Island, NY), pH 7.2-7.4, supplemented with 500 U/ml of recombinant murine GM-CSF (R&D Systems, Minneapolis, MN) and 1,000 U/ml of recombinant murine IL-4 (PharMingen, San Diego, CA), and incubated in a 5% CO_2 atmosphere at 37°C for a total of four to five days. The medium was replaced on day two with additional recombinant cytokines.

LPS Stimulation of DCs

DCs were stimulated on day four with varying doses of LPS (*Escherichia coli 055:B4*), ranging from 0.1 μg/ml to 10 μg/ml and supplemented with recombinant

cytokines. DCs were collected for flow cytometric analysis, and supernatant was collected for cytokine analysis at various times ranging from two to 24 hours.

Cowpox Virus Infection of DC

DCs were incubated on day four with varying quantities of the Brighton-Red strain of cowpox virus (CPV) for two hours in serum-free media. DCs were infected with either wild-type cowpox virus or recombinant cowpox virus expressing green fluorescent protein (GFP). The recombinant cowpox virus expressing GFP under a CMV promoter was produced according to Stern *et al.* [32]. The number of infectious particles ranged from 10^5 to 10^7 particles/ml for 10^6 DCs, yielding approximate MOIs from 0.1 to 10. Thereafter, the plate was centrifuged (Beckman GPR centrifuge) at 1,000 rpm for five minutes and the media was completely replaced and supplemented with recombinant GM-CSF and IL-4. DCs were incubated in complete media for periods between six and 24 hours and then harvested for flow cytometric analysis, or cytokine secretion. For experiments in which maturation of DCs was subsequently examined after poxvirus infection, 10 μg/ml of LPS was added after the six hour infection, for varying amounts of time.

Flow Cytometric Analysis of DCs

Six hours following poxvirus infection with or without stimulation by LPS, cells were harvested and centrifuged at 1,200 rpm for five minutes. The supernatant was collected and frozen at -70° C for cytokine analysis. The cell pellet was washed with 1% flow buffer (1% bovine serum albumin (BSA), 1mM EDTA (Fisher Scientific, Atlanta, GA) and 0.1% sodium azide (NaN_3: Sigma Chemical Co., St. Louis, MO) in Hanks' balanced salt solution without phenol red, calcium and magnesium (Cellgro, Herndon, VA)), and resuspended in 500 μl of 1% paraformaldehyde solution (25% Formaldehyde

(FischerChemical, Fair Lawn, NJ) in 1% flow buffer) to fix the cell membrane and inactivate any active viral particles. Prior to flow cytometric analysis, cells were resuspended in 4% BSA flow buffer and blocked with CD16/CD32 Fc antibodies (PharMingen, San Diego, CA) followed by staining, as described below. DCs were identified using anti-CD11c and anti-CD8 antibodies, and DC maturation was determined based on the relative surface expression of CD86 and MHC Class II (Immunotech, Miami, FL). Antibodies were either directly conjugated with PE (PharMingen, San Diego, CA), or indirectly conjugated to an anti-biotinylated antibody labeled with APC (Molecular Probes, Eugene, OR). Samples were analyzed on a FACSCalibur® instrument with Lysis II® Software (Becton Dickinson Systems, San Jose, CA).

Infectivity Assessment via Flow Cytometry

Infection efficiency of DCs was assessed using a recombinant cowpox virus/GFP (CPV/GFP) construct (provided as a gift by Dr. Pete Turner, Department of Molecular Genetics and Microbiology). DCs were infected at an MOI of 10 as previously outlined, then collected and fixed in 1% paraformaldehyde. Cells expressing GFP were quantified on a FACSCalibur® instrument to determine the rate of infection.

Cytokine Measurements

DC culture supernatants were collected and measured for concentrations of murine TNFα, IL-12p70 and IL-10 by specific ELISA using commercially available reagents (Endogen for TNFα, R&D Systems for IL-10, and BD-Pharmingen, Inc. for IL-12p70).

Analysis of Cell Viability

DCs were infected for two hours at various MOI and then allowed to incubate for various amounts of time ranging from two to twenty four hours. DCs were then treated with 6 mg/ml yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-

diphenyltetrazolium bromide) for four hours, lysed with isopropanol and read on the VERSAmax™ microplate reader.

Statistical Analyses

Data are presented as the mean ± S.E.M. Differences among treatment groups were determined by analysis of variance, after passing tests of normality. In those cases, where normality was not confirmed, differences among groups were compared using the Kruskal-Wallis ANOVA on ranks. Post hoc comparisons against the controls were performed using the Dunnett's multiple range test. In some cases, a pair t-test against controls was employed with Bonferroni's correction for multiple comparisons. In all cases, significance was determined at the 95% confidence level.

CHAPTER 3
RESULTS

There were three specific aims to the study: 1) to determine whether poxvirus directly infected myeloid-derived murine DCs, and whether these infections were cytotoxic; 2) to determine whether infection with poxvirus induced DC maturation, and this maturation was dependent on cell infection, or resulted from a bystander effect on locally infected cells; and, 3) whether infection with poxvirus altered the ability of DCs to respond to a subsequent prototypical inflammatory stimulus, induced by LPS.

Rate of Infection Was Determined Using a CPV/GFP Vector

Myeloid derived DCs were generated from mixed cell populations obtained from the bone marrows of C57BL/6 mice. After culturing these cells for four days with IL-4 and GM-CSF, cells were infected with increasing doses of a recombinant CPV expressing GFP. The percentage of DCs infected with the CPV/GFP was analyzed by flow cytometry. As shown in Figure 2 (n=12), at MOIs approximating 10, approximately 46% of the $CD11c^+$ cells were expressing GFP, indicative of an active infection.

Cell Death from CPV at an MOI of 10 Occurs within 24 Hours

DC viability was analyzed at two, six and 24 hours after infection with wild-type CPV at a MOI of 0.1, 1, and 10 pfu/cell. Cell viability was determined by the reduction of the chromophore MTT, determined in 96 well microtiter plates. Because the assay was performed in a 96 well plate, we could not distinguish cell viability between infected

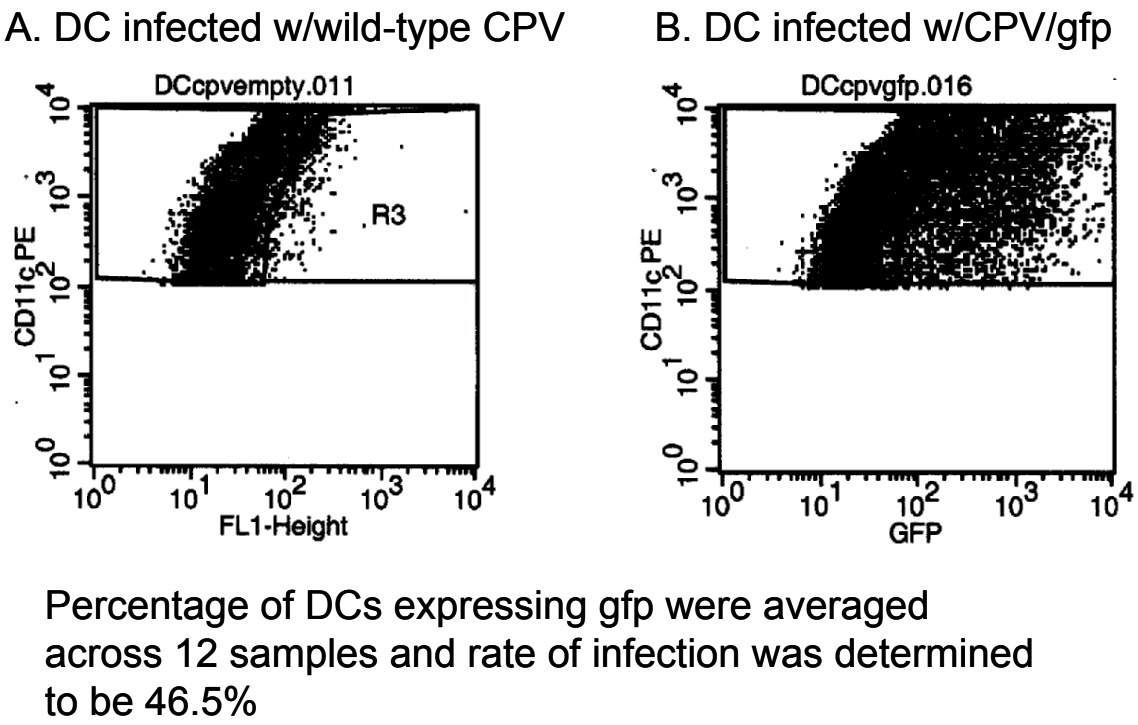

Figure 2. Determination of infectivity rate using CPV/GFP vector

Note: Immature DCs ($CD11c^+$) were infected with either wild-type cowpox virus or CPV/GFP at MOI of 10 for six hours, as outlined in the Materials and Methods section. DCs were harvested and stained for flow cytometry using biotinylated antibodies to $CD11c^+$ and analyzed on a FACSCalibur® instrument. $CD11c^+$ cells staining positive for GFP were designated as cells positive for cowpox virus infection.

cells and those incubated with poxvirus but not infected. As shown in Figures 3-5, significant reductions in cell viability were not observed until six hours after infection with an MOI of 10. There was some decrease in cell viability at 24 hours with infections at an MOI of 1. However, at the highest infection (MOI of 10 pfu/cell) at 24 hours, cell viability declined 55% suggesting that cytolysis was similar to infectivity (46%).

LPS Is a Potent Stimulator of DC Maturation

The second specific aim was intended to examine whether infection with cowpox virus induced DC maturation, and whether maturation was dependent upon cell infection. To establish a positive control for DC maturation, immature DCs were stimulated with

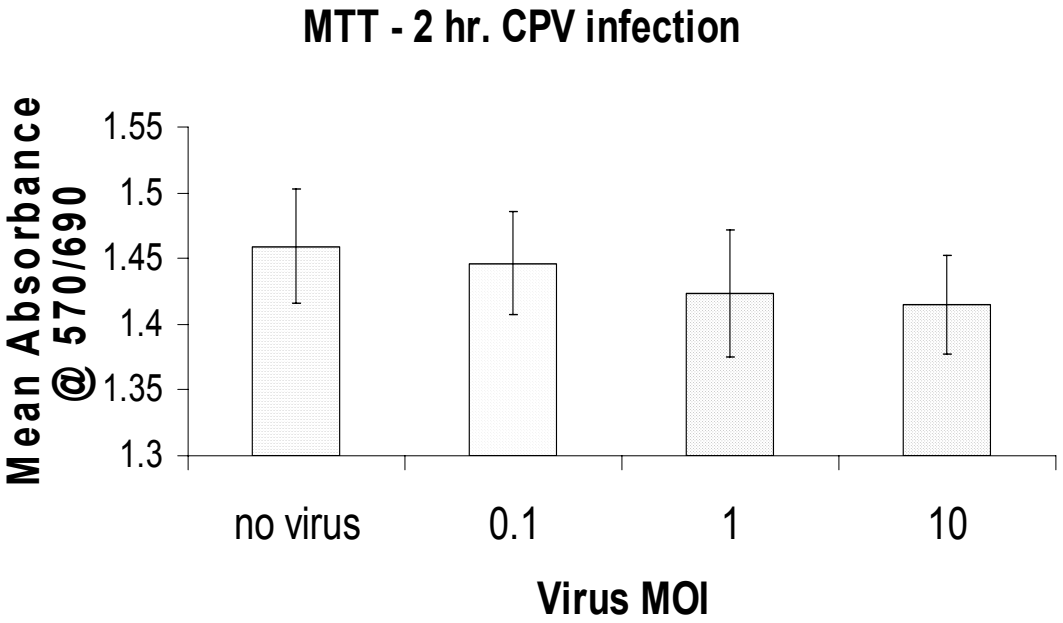

Figure 3. Analysis of cell viability after 2 hr. of cowpox virus infection.

Note: DCs were infected at MOI- 0.1, 1, 10 of CPV, or not infected for two hours, and the virus was removed by washing the plates. The cells were then allowed to incubate for an additional two hours. DCs were then treated with MTT for four hours, lysed with isopropanol and water, and absorbance was recorded at 570/690nm. There was no difference in cell viability after two hours following infection with varying MOIs of cowpox virus. Values represent the mean of eight replicates.

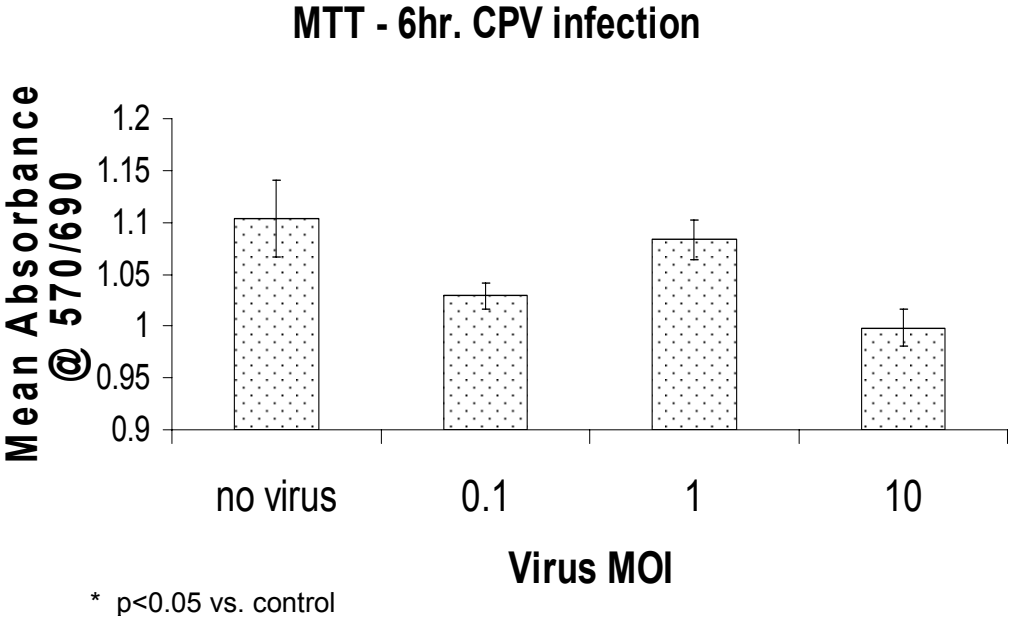

Figure 4. Analysis of cell viability after 6 hr. cowpox virus infection

Note: DCs were infected at MOI- 0.1, 1, 10 of cowpox virus, or not infected, as described in Figure 3, and allowed to incubate for six hours. DCs were then treated with MTT for four hours, lysed with isopropanol and absorbance was recorded at 570/690nm. Data were analyzed by two way ANOVA and Dunnett's multiple range test. An asterisk (*) indicates that the cell viability, as determined by MTT reduction, was reduced in the group of cells treated with an MOI of 10 cowpox virus, at the $p<0.05$ confidence level. Values represent the mean of eight replicates.

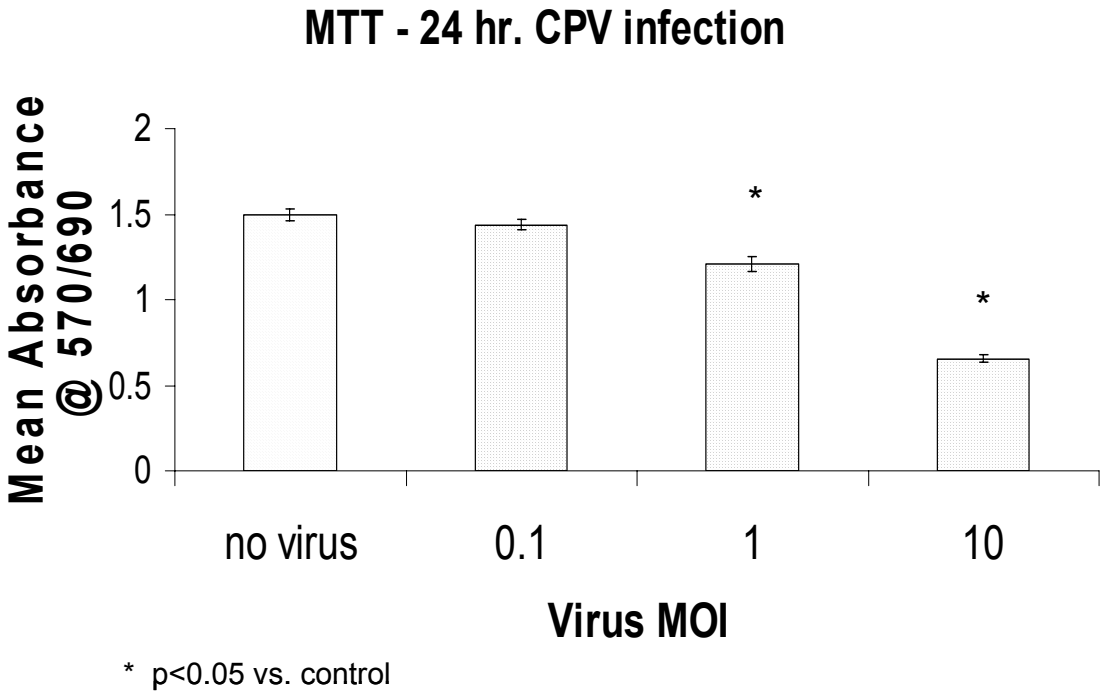

Figure 5. Analysis of cell viability after 24 hr. cowpox virus infection

Note: DCs were infected at MOI- 0.1, 1, 10 of CPV, or not infected, as described in Figure 3, and allowed to incubate for 24 hours. DCs were then treated with MTT for four hours, lysed with isopropanol and absorbance was recorded at 570/690nm. Data were analyzed by two way ANOVA and Dunnett's multiple range test. An asterisk (*) indicates that the cell viability, as determined by MTT reduction, was reduced in the group of cells treated with an MOI of 1 and 10 cowpox virus, at the $p<0.05$ confidence level. Values represent the mean of eight replicates.

bacterial LPS, a potent TLR4 ligand and activator of innate immunity [33]. A number of criteria were used to assess the maturation status of the DC population, including the cell surface expression of MHC class II and the costimulator molecule CD86. As shown in Figure 6, cell surface expression was determined by flow cytometry, and $CD11c^+$ cells

were gated for MHC class II and CD86 expression. A representative contour plot is shown where $CD11c^+$ cells were additionally stained with FITC labeled MHC class II and APC labeled CD86 antibodies. Mature DCs were identified by the proportion of cells who were high expressers of both MHC class II and CD86. The summary results are presented in Table 1. With 10 μg/ml of LPS as a stimulant, DCs expressing both MHC-II and CD86 increased from 24.1% to 41.3% ($p<0.05$).

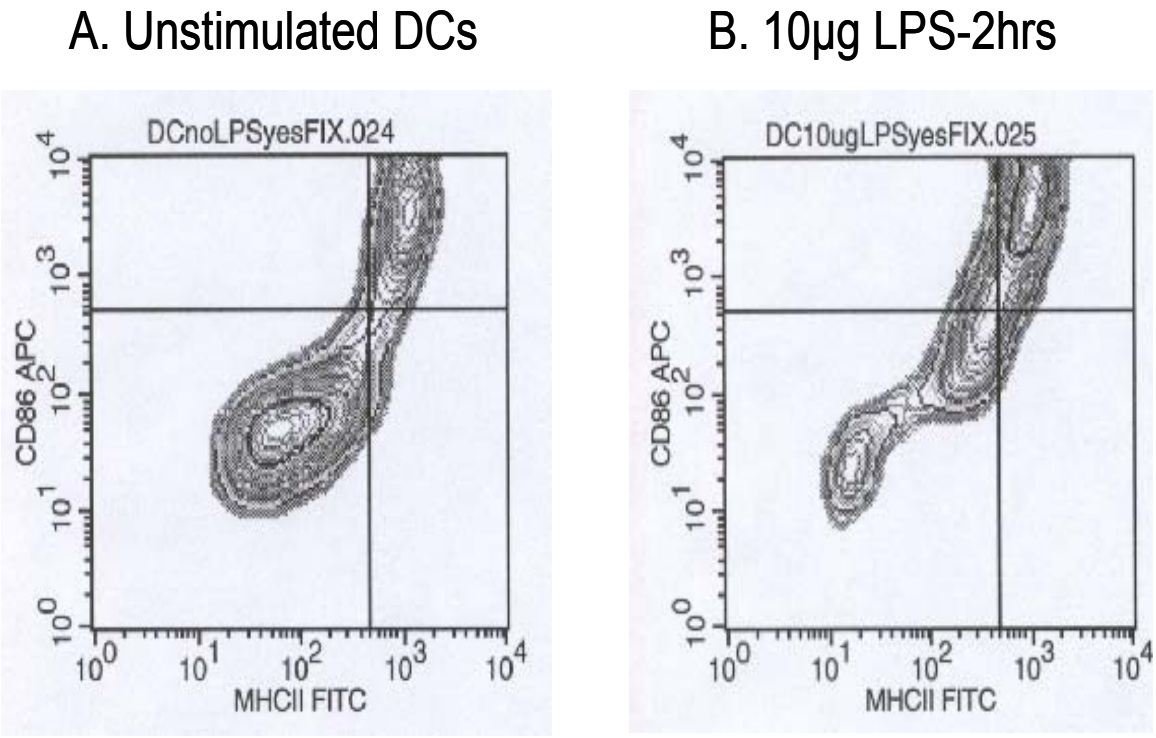

Figure 6. Contour plot showing the effect of LPS stimulation on CD86 and MHC Class II surface expression.

Note: GM-CSF and IL-4 generated DCs were stimulated with 10 μg LPS for 24 hrs and harvested as outlined in the Materials and Methods section. DCs were then stained with conjugated antibodies to CD11c, CD86, and, MHC class II. Cell debris was gated out by omitting all signals that were outside the forward and side scatter channels. CD11c+ cells were gated, and then analyzed for surface expression of MHC-II and its costimulatory molecule, CD86, as a measure of maturation. The x-axis represents the intensity of cell staining for MHC class II, and the y-axis represents the intensity of staining for CD86. Panel A is representative analysis of unstimulated DCs while Panel B is a representative analysis of LPS stimulated DCs. It can be inferred that cells within the upper right-hand quadrant (high expression for both MHC class II and CD86) exhibit a

mature phenotype, and cells that lie within the lower left-hand quadrant exhibit an immature phenotype (MHC class II and CD86 low).

Table 1- Summary of DC maturation from C57BL/6, TNFR I null and IL-10 null mice following stimulation with LPS.

		B6			TNFR k/o			IL-10 k/o	
	No LPS	**1ug**	**10ug**	**No LPS**	**1ug**	**10ug**	**No LPS**	**1ug**	**10ug**
Exp. 1	31.53	39.62	39.34	24.06	41.36	41.33			
	24.08	38.28	40.32	25	39.62	40.45			
Exp. 2	30.41	54.68	59.16	43.25	53.09	57.92	39.58	63.33	64.35
	41.76	62.56	59.87	45.36	52.79	58.92	35.24	63.11	64.88
Mean	**31.945**	**48.785**	**49.6725**	**34.4175**	**46.715**	**49.655**	**37.41**	**63.22**	**64.615**
					*	*			
Std. Dev.	7.319401	11.816	11.37587	11.45598	7.224064	10.13555	3.068843	0.155563	0.374767
Std. Err.	3.659701	5.908002	5.687937	5.72799	3.612032	5.067775	2.17	0.11	0.265

* $p<0.05$ vs. no LPS

Note: Following 24 hr. stimulation with either nothing, 1 μg LPS, or 10 μg LPS, cells were harvested and stained for flow cytometry as outlined in the Materials and Methods section. Mature DCs were then identified using the same gating criteria as previously described. Percentages of mature DCs were calculated and compared among treatment groups of the three different strains of mice. Values represent the mean of either four or two replicates. An asterisk (*) indicates that the differences in percent maturation versus unstimulated controls was significant at the $p<0.05$ level of confidence, as determined either by ANOVA (TNFR I knockout, or paired t-test with Bonferroni's correction; C57BL/6)

In addition to cell surface molecule expression, the activation or maturation status of DCs was evaluated by the release of Th1 and proinflammatory cytokines, TNF-α and IL-12p70, as well as the anti-inflammatory cytokine, IL-10 at 24 hours after stimulation. As shown in Figures 7-9, LPS stimulated DCs to produce increased quantities of both TNF-α and IL-12 over the first 24 hours, but did not increase the production of IL-10. Caution must be used in interpreting these results since they represent a single replicate. Additional replicates are presented in later tables when the maturation response to LPS is directly compared to cowpox virus infected cells.

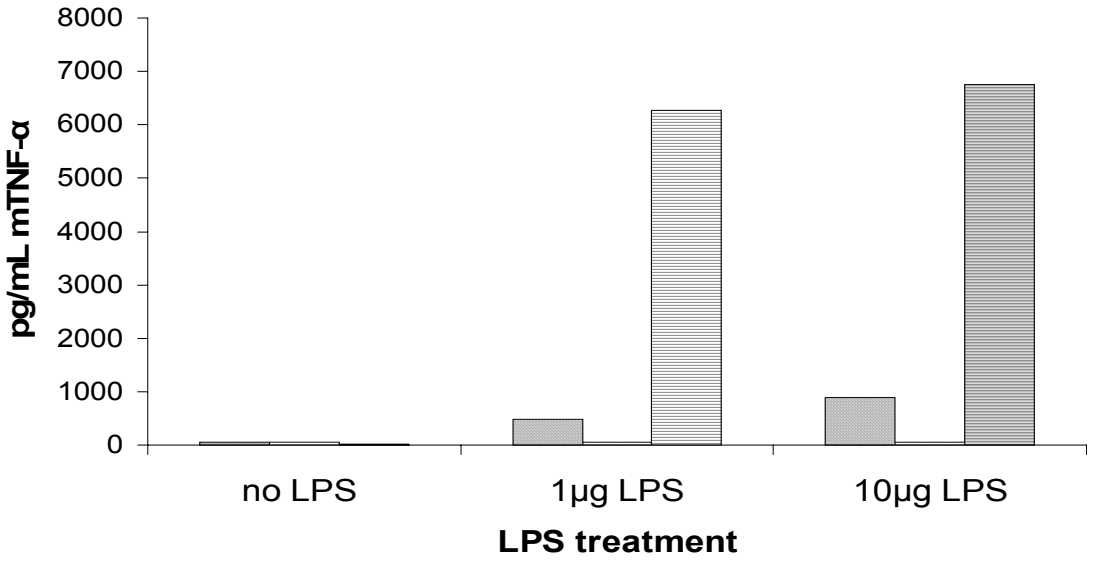

Figure 7. TNF-α production in DCs from C57BL/6 wild type, sTNFR-I null, and IL-10 null mice when stimulated with LPS.

Note: Following a 24 hr stimulation with either nothing, 1 µg LPS, or 10 µg LPS, cells were harvested and supernatant was collected as outlined in the Materials and Methods section. TNF-α concentrations were determined by ELISA. ▨ represents DCs obtained from C57BL/6 mice; ⁘ DCs obtained from TNFR-I null mice, and ▤ DCs obtained from IL-10 null mice. Values of zero indicate that TNF-α levels were below detectable limits. Values represent a single replicate. No statistical analyses were performed.

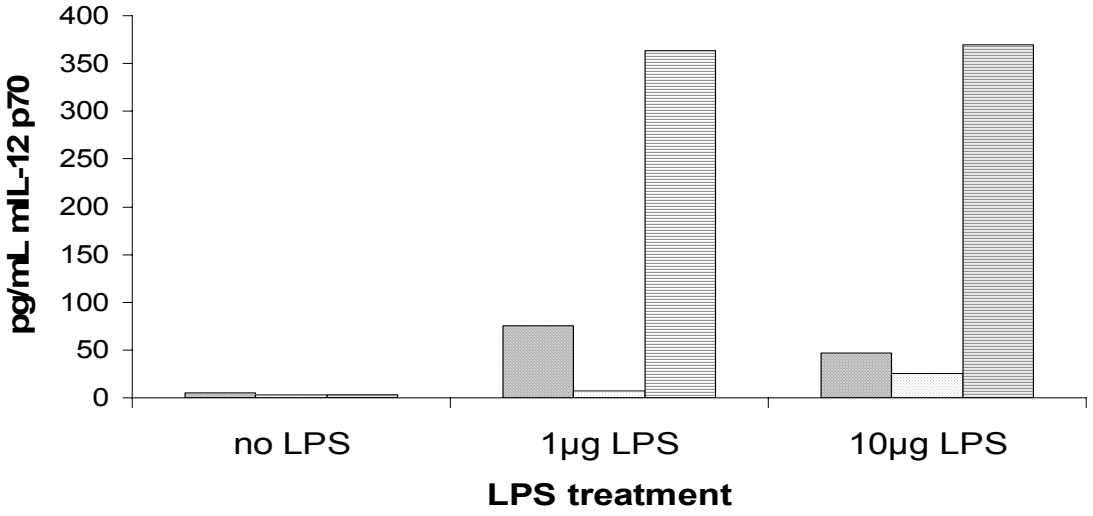

Figure 8. mIL-12 p70 production in DCs from C57BL/6 wild type, sTNFR-I null, and IL-10 null mice when stimulated with LPS.

Note: Following 24hr. stimulation with either nothing, 1 μg LPS, or 10 μg LPS, cells were harvested and supernatant was collected as outlined in the Materials and Methods section. IL-12 concentrations were determined by ELISA. ▨ represents DCs obtained from C57BL/6 mice; ⁘ DCs obtained from TNFR-I null mice, and ▤ DCs obtained from IL-10 null mice. Values of zero indicate that IL-12 levels were below detectable limits. Values represent a single replicate. No statistical analyses were performed.

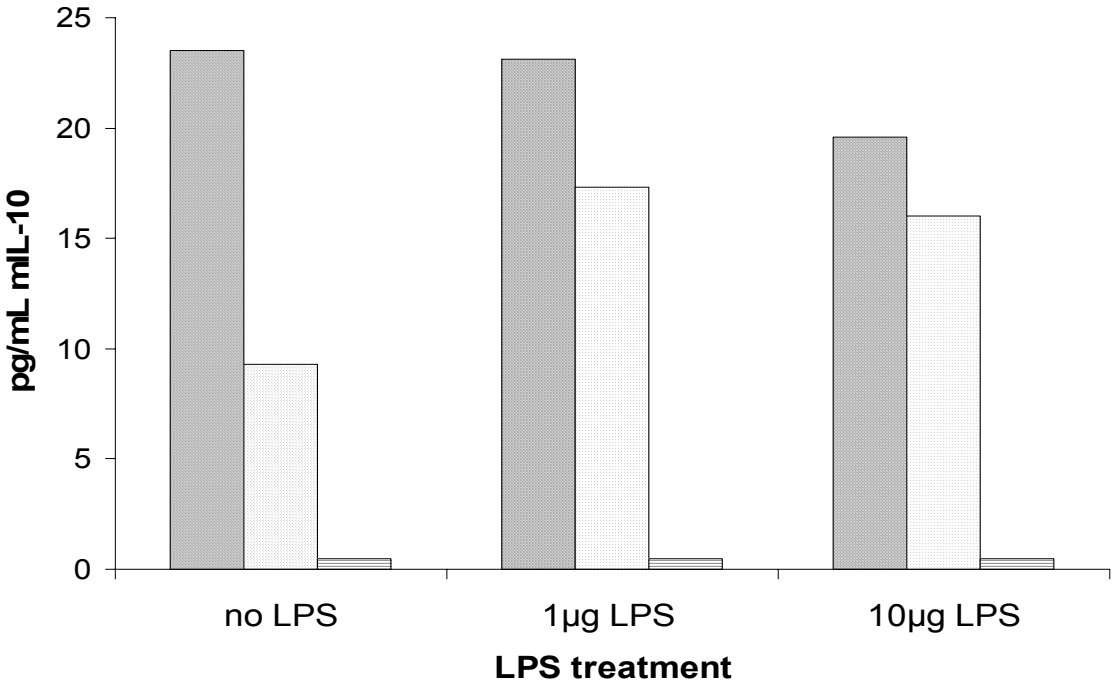

Figure 9. IL-10 production in DCs from C57BL/6, sTNFR-I null, and IL-10 null mice when stimulated with LPS.

Note: Following 24 hr. stimulation with either nothing, 1 μg LPS, or 10 μg LPS, cells were harvested and supernatant was collected as outlined in the Materials and Methods section. ▨ represents DCs obtained from C57BL/6 mice; ⁘ DCs obtained from TNFR-I null mice, and ▤ DCs obtained from IL-10 null mice. Values of zero indicate that IL-10 levels were below detectable limits. Values represent a single replicate. No statistical analyses were performed.

DC Maturation Proceeds Normally in TNF Receptor Type-I Null Mice and in an Exaggerated Pattern in IL-10 Null Mice

As an initial effort to determine the dependence of endogenous TNF-α and IL-10 signaling on DC maturation in response to LPS, these studies were also carried out with DCs obtained from TNFR-I and IL-10 null mice. Because these were preliminary studies and were not the primary focus of the experiment, the studies looking at cell surface expression were replicated only two times (n=4), whereas the cytokine measurements were determined only once. As shown in the contour plot in Figure 10, $CD11c^+$ cells from TNFR I null mice responded to LPS stimulation with a response similar to DCs from wild type animals with marked increases in both CD86 and MHC class II expression. Studies from the Moldawer laboratory have previously shown that lethality to endotoxicosis is dependent upon TNF signaling through the TNFR I receptor [34]. These data are further summarized in Table 1 and Figures 7-9. Surprisingly, DCs from TNFR I null mice had barely detectable TNF-α release, suggesting that TNF signaling is required for amplification of TNF-α production in response to LPS. Furthermore, IL-12p70 production in response to LPS was dramatically reduced, especially at the lower dose of LPS, suggesting that this response was also dependent to some extent on TNF signaling. Interestingly, DCs from IL-10 knockout mice showed both an exaggerated expression of cell surface molecules, and dramatically increased levels of TNFα and IL-12, consistent with its role as an endogenous anti-inflammatory agent [35].

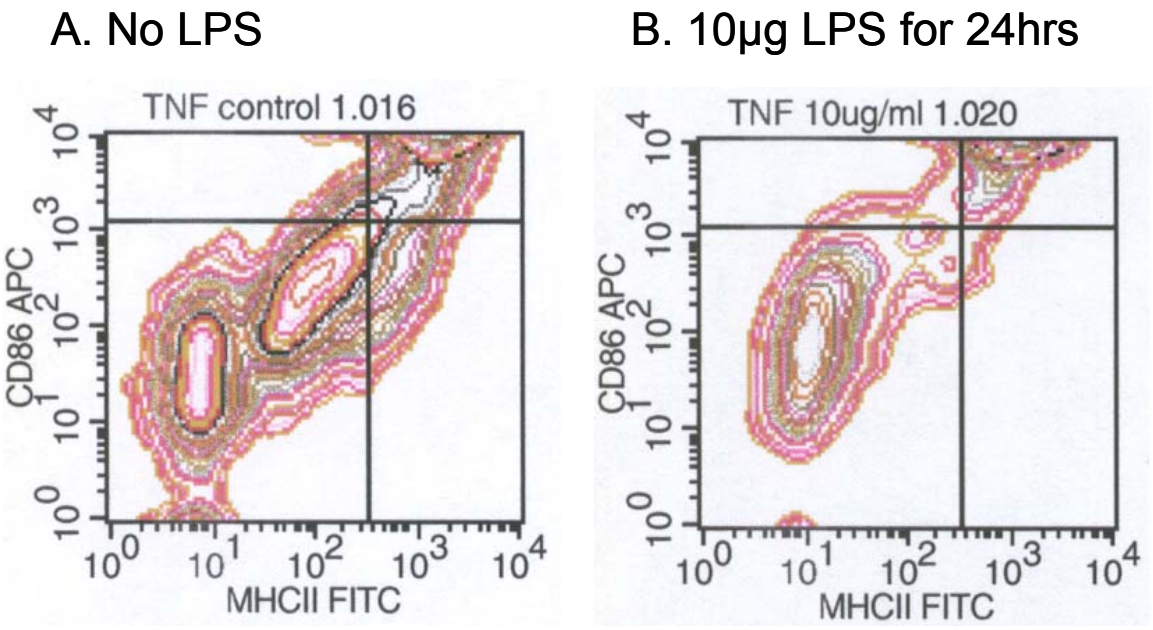

Figure 10. Contour plot showing CD86 and MHC class II expression of DCs from TNFR I null mice stimulated with LPS.

Note: DCs were stimulated with 10 µg LPS for 24 hrs and harvested as outlined in the Materials and Methods section. DCs were then stained with fluorescent conjugated antibodies to CD11c, CD86, and, MHC class II. Cell debris was gated out by omitting all signals that were outside the forward and side scatter channels. Next, cells were gated on $CD11c^+$ expression, and the resulting positive cell population was then analyzed for surface expression of MHC class II and its costimulatory molecule, CD 86, as a measure of maturation. The x-axis represents the signal intensity for MHC class II, and the y-axis represents the signal intensity for CD86. Panel A represents DCs not stimulated with LPS while Panel B represents DCs stimulated with 10 µg/ml of LPS. All cells that lie within the upper right-hand quadrant are designated as exhibiting a mature phenotype ($MHC\ II^{high}$ and $CD86^{high}$) and all cells that lie within the lower left-hand quadrant exhibit an immature phenotype.

Cowpox Virus Does Not Induce DC Maturation

DCs infected with cowpox virus displayed dramatically different cell surface expression and cytokine production profiles than did DCs stimulated with LPS alone. As shown in the contour plot in Figure 11, cowpox virus infection did not induce any increase in the expression of MHC class II or CD86. With doses as high as 10 MOI,

there was no increase in cell surface expression, despite a significant increase in cell death at 24 hours.

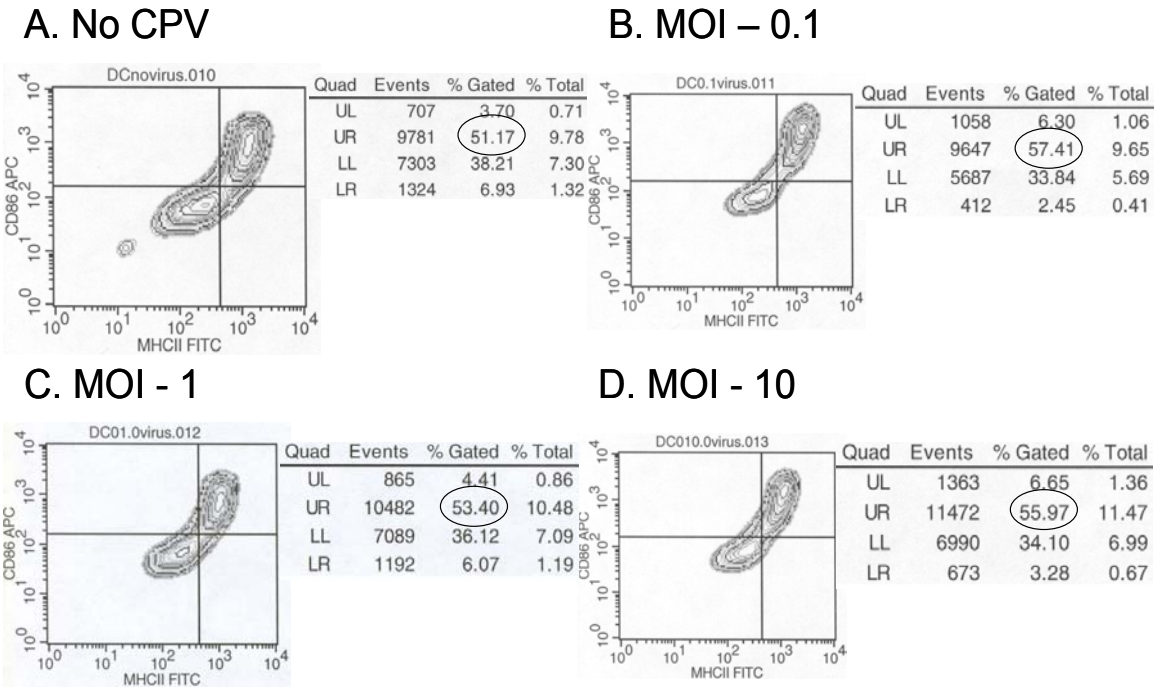

Figure 11. Contour plot showing DC maturation in response to cowpox virus.

Note: Cultured DCs were infected with cowpox virus at MOI of 0.1, 1, and 10 for two hours, washed them and allowed to incubate for an additional 6 hrs. The percentage of DCs exhibiting a mature phenotype was then compared to cultured DCs that were not infected with CPV. DCs were stained with conjugated antibodies to CD11c, CD86, and, MHC-II as outlined in the Materials and Methods section. Cell debris was gated out by omitting all signals that were outside the forward and side scatter channels. Next, cells were gated on $CD11c^{+}$, and the resulting cell population was then analyzed for surface expression of MHC-II and its costimulatory molecule, CD86, as a measure of maturation. he x-axis represents the signal intensity for MHC class II, and the y-axis represents the signal intensity for CD86. Panel A represents DCs not stimulated with cowpox virus while Panel B represents DCs stimulated with 0.1 MOI of cowpox virus, Panel C with 1 MOI of cowpox virus and Panel D with 10 MOI of cowpox virus. All cells that lie within the upper right-hand quadrant are designated as exhibiting a mature phenotype (MHC II^{high} and $CD86^{high}$) and all cells that lie within the lower left-hand quadrant exhibit an immature phenotype. As demonstrated here, cowpox infection was not associated with any increase in the percentage of cells with a mature phenotype.

Cowpox Virus Inhibits DC Ability to Respond to LPS following an Infection

To examine whether DCs infected with cowpox virus could respond to a subsequent inflammatory challenge like LPS, DCs infected with 10 MOI of cowpox virus were challenged after six hours with 10 μg of LPS. To distinguish the direct effects of cowpox virus on infected cells, from cells incubated with virus but not infected, a recombinant cowpox virus expressing GFP was employed, and GFP positive (indicative of infection) and GFP negative DCs were gated to examine their cell surface expression. Looking at the DCs as a group in their entirety, MHC class II expression was increased in all DCs exposed to LPS (at a significance level of $p=0.07$, by two-way ANOVA), regardless of whether they had been pre-exposed to cowpox virus (Figure 12). By gating on the GFP expressing cells, we could subdivide the analysis into cells directly infected by cowpox virus and those exposed presumably to a bystander effect. There was no difference in the MHC class II expression induced by LPS in cells directly infected with cowpox virus and those not infected, although cells expressing GFP tended to have higher MHC class II expression than cells not expressing GFP (Figure 13).

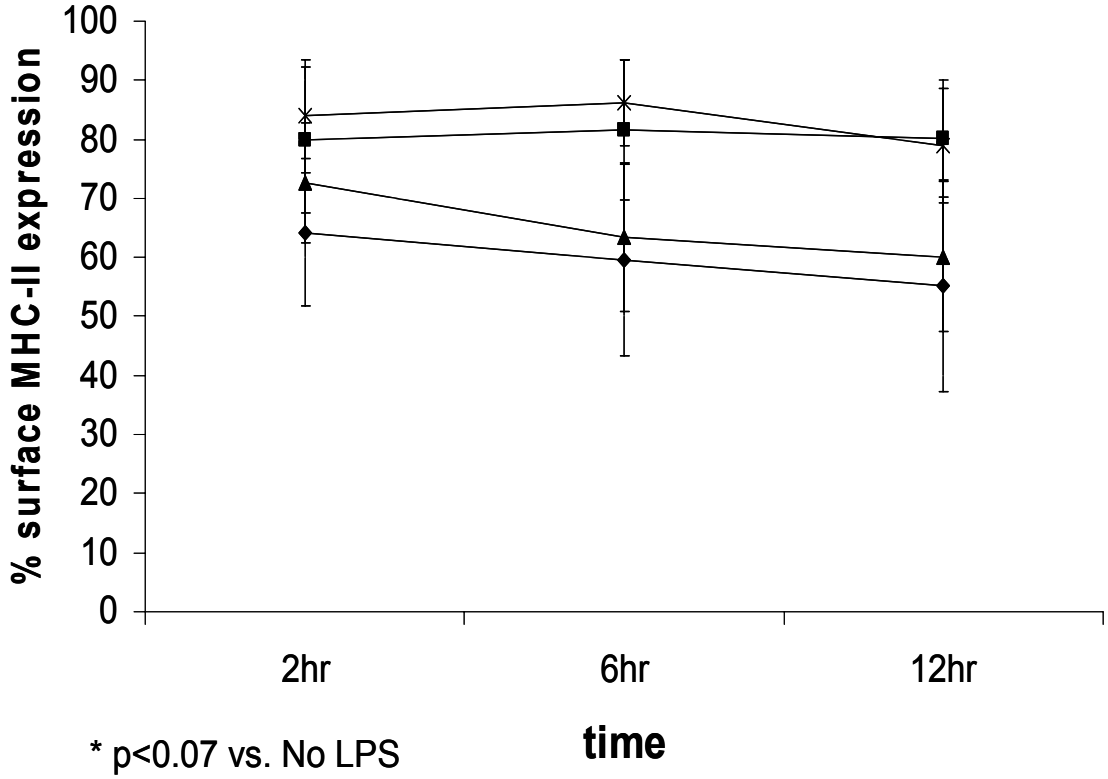

Figure 12. Flow cytometric analysis of MHC-II expression in DCs stimulated with LPS following a 6hr. cowpox infection.

Note: DCs were infected with recombinant cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for 2, 6, and 12 hrs. as outlined in the Materials and Methods section. DCs were harvested and stained for flow cytometry as outlined in the Materials and Methods section. GFP+ cells were recognized as cells positive for cowpox virus infection. Biotinylated antibodies to MHC II were detected using APC and analyzed on a FACSCalibur® instrument. X---X indicates cowpox virus infection followed by LPS stimulation, ■----■ LPS stimulation alone, ▲------▲ cowpox virus infection alone, and ♦-----♦ no treatment. Data represent the mean of three replicates, and were analyzed by two way, ANOVA. The differences in MHC class two expression among the four groups was p=0.07.

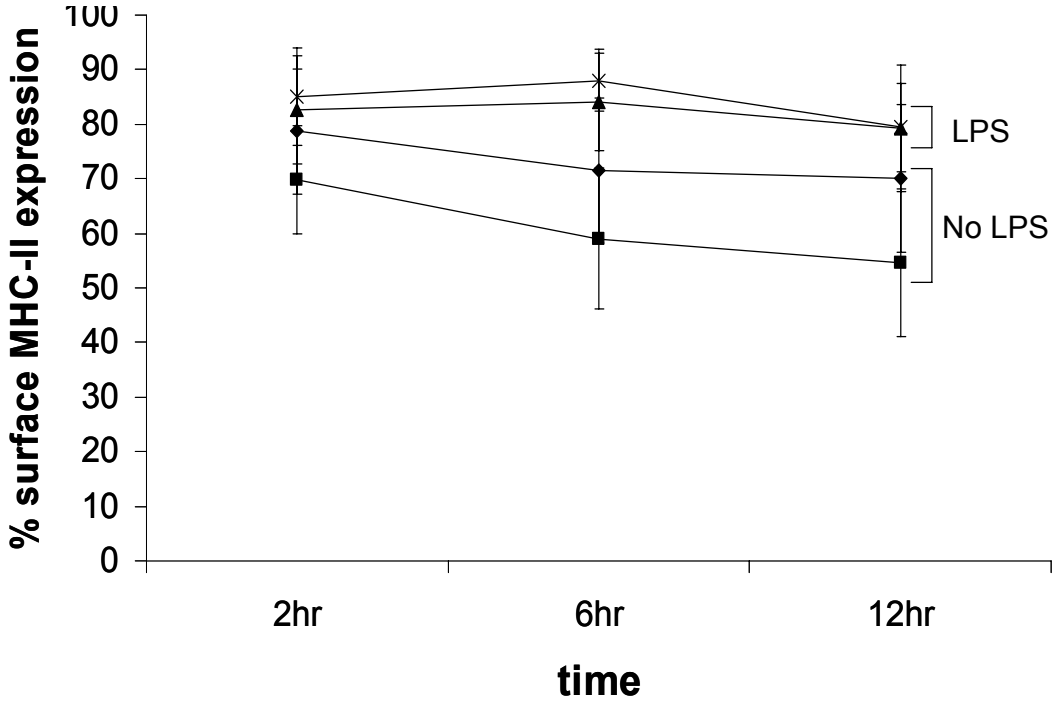

Figure 13. Flow cytometric analysis of MHC-II expression in GFP expressing vs. nonexpressing DCs stimulated with LPS following a 6 hr. cowpox virus infection.

Note: DCs were infected with cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for 2, 6, and 12 hrs. as outlined in the Materials and Methods section. GFP+ cells were considered to be cells positive for cowpox virus infection. Biotinylated antibodies to MHC-II were detected using APC and analyzed on a FACSCalibur® instrument. ♦----♦ positive for infection by cowpox virus with no LPS stimulation, ■---- ■ negative for infection by cowpox virus with no LPS stimulation, ▲----▲ positive for infection with CPV followed with LPS, X----X negative infection of CPV followed with LPS.

Interestingly, the CD86 response was very different. When DCs were infected with cowpox virus for six hours and then stimulated with LPS for varying amounts of time, CD86 expression was significantly less than seen in cells stimulated with LPS alone (Figure 14). While CD86 expression in this treatment group was higher than the cowpox virus infection only treatment group, these results suggest that the virus infection has clearly reduced the DC's ability to express surface CD86. Interestingly, this reduction in

class II expression in cowpox virus infected cells stimulated with LPS appeared to affect both the cells actually infected and those not infected, but experiencing a bystander effect. (Figure 15) Thus, it appears that the suppression in LPS induced CD86 expression by CPV may not be due to a direct cytolytic effect of the virus, but rather secondary to humoral factors released by the cells.

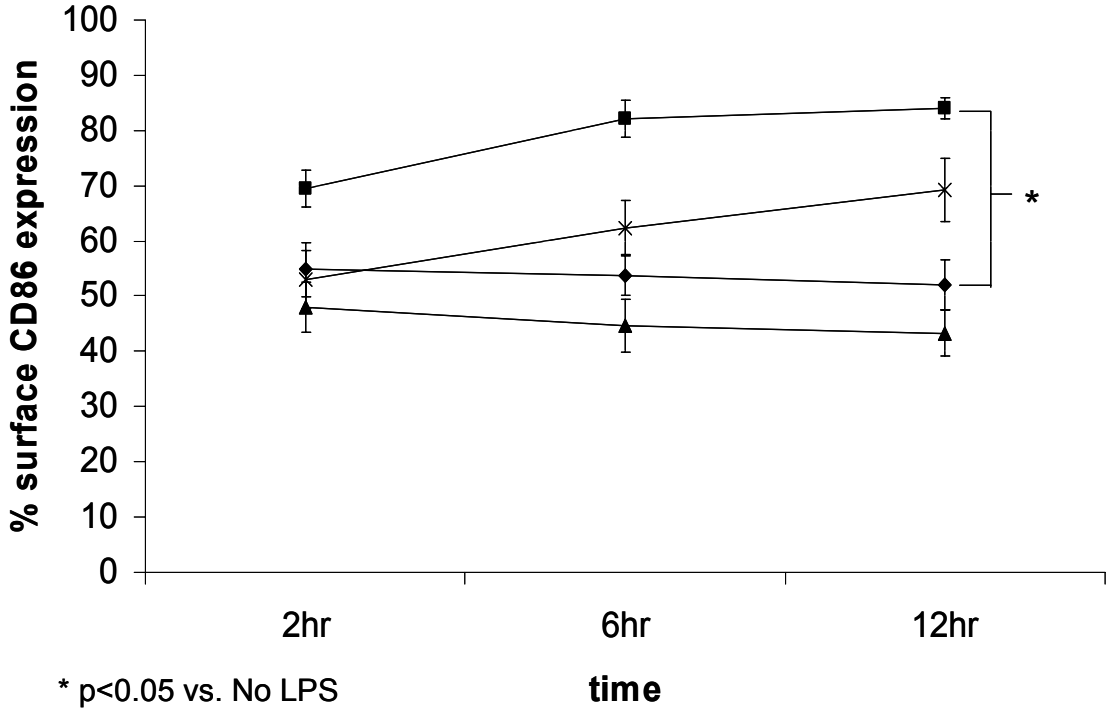

Figure 14. Flow cytometric analysis of CD86 expression in DCs stimulated with LPS following a 6 hr. cowpox infection.

Note: DCs were infected with recombinant cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for 2, 6, and 12 hrs. as outlined in the Materials and Methods section. DCs were harvested and stained for flow cytometry as outlined in the Materials and Methods section. GFP+ cells were recognized as cells positive for cowpox virus infection. Biotinylated antibodies to CD86 were detected using APC and analyzed on a FACSCalibur® instrument. X---X indicates cowpox virus infection followed by LPS stimulation, ■----■ LPS stimulation alone, ▲------▲ cowpox virus infection alone, and ♦-----♦ no treatment. Data represent the mean of three replicates, and were analyzed by two way, ANOVA. The asterisk (*) indicates that the CD86 expression in the LPS treated cells were different than controls ($p<0.05$). Prior infection with cowpox virus attenuated the CD86 expression to subsequent LPS stimulation.

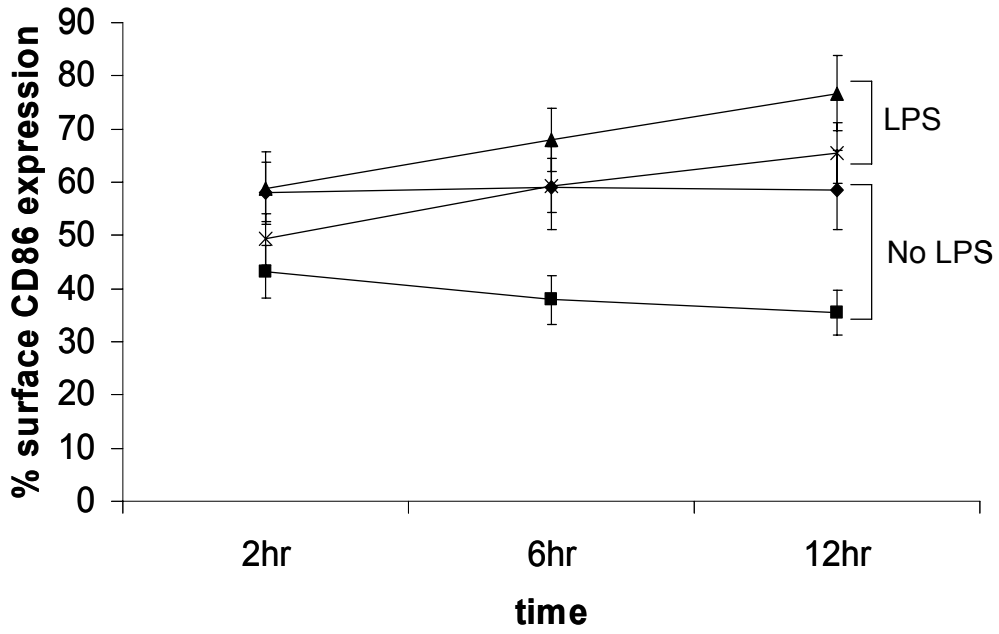

Figure 15. Flow cytometric analysis of CD86 expression in GFP expressing vs. nonexpressing DCs stimulated with LPS following a 6 hr. cowpox virus infection.

Note: DCs were infected with cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for 2, 6, and 12 hrs. as outlined in the Materials and Methods section. GFP+ cells were considered to be cells positive for cowpox virus infection. Biotinylated antibodies to CD86 were detected using APC and analyzed on a FACSCalibur® instrument. ♦----♦ positive for infection by cowpox virus with no LPS stimulation, ■---- ■ negative for infection by cowpox virus with no LPS stimulation, ▲----▲ positive for infection with CPV followed with LPS, X---X negative infection of CPV followed with LPS. There was no difference in the CD86 expression between GFP+ and GFP- expressing cells stimulated with LPS or not, although CD86 expression tended to be higher in the GFP+ cells. These findings suggest that the effect of cowpox virus infection was observed regardless of whether the cells were GFP expressing.

As shown in Figures 16-18, prior infection with cowpox virus also dramatically altered the pattern of cytokine production in DCs treated with LPS. For example, the production of TNF-α and IL-12 were significantly reduced in DCs that were infected with cowpox virus. IL-10 levels were unaffected.

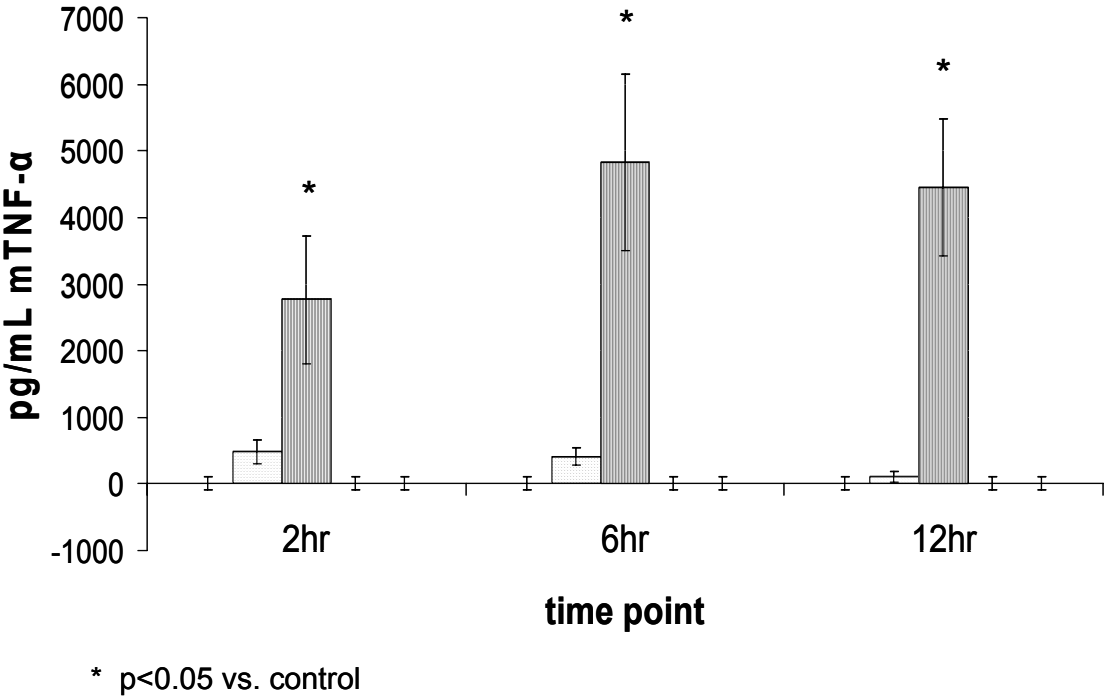

Figure 16. TNF-α production by DCs stimulated with LPS following a 6 hr. cowpox infection (CPV/GFP).

Note: DCs were infected with a recombinant cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 □g LPS for two, six, and 12 hrs, as outlined in the Materials and Methods section. Additionally, some DCs were infected with cowpox virus at an MOI of 10 in the presence of 1 □g/ml of anti-mIL10 antibody in culture. TNF-α was determined by ELISA. ▨ DCs infected with CPV alone, ⁝⁝ DCs infected with CPV followed by LPS, ▥ DCs stimulated with LPS alone, ▦ no treatment, and ▤ DCs stimulated with CPV in the presence of anti-IL10. Values of zero indicate that TNF-α levels were below detectable limits. Values represent the mean of three replicates each (with the exception of the anti-IL-10 studies which were repeated only once). An asterisk (*) indicates that the differences in TNF-α production in response to the LPS was significantly different than from no treatment controls, as determined by two way ANOVA and Dunnett's multiple range test. It is important to note that the TNF-α production by DCs infected with cowpox virus and then stimulated with LPS was not different than no treatment controls.

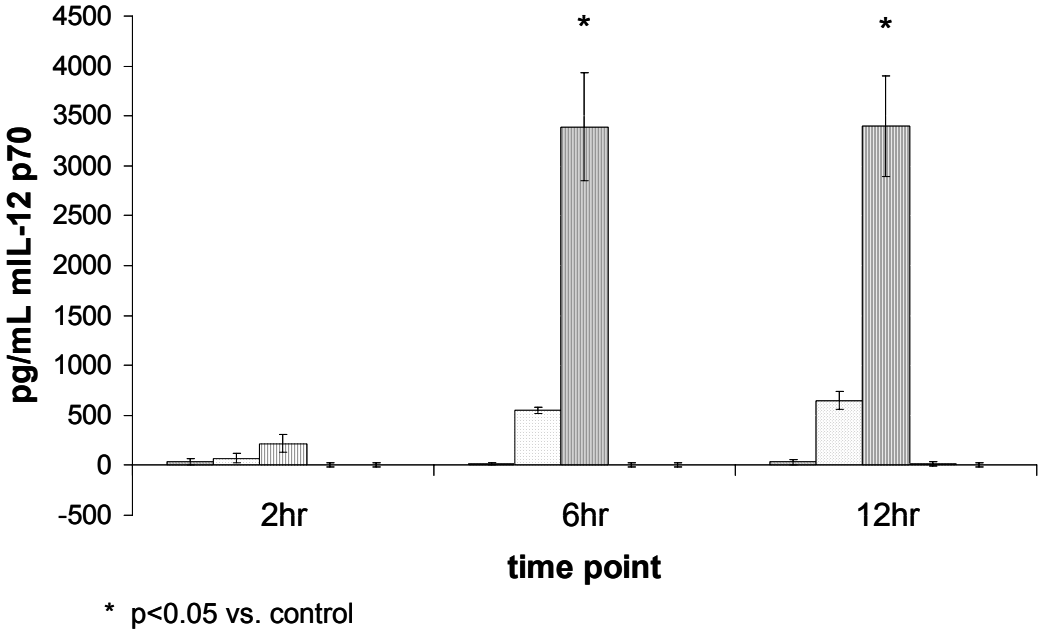

Figure 17. IL-12 p70 production by DCs stimulated with LPS following a 6 hr. cowpox infection.

Note: DCs were infected with a recombinant cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for two, six, and 12 hrs, as outlined in the Materials and Methods section. Additionally, some DCs were infected with cowpox virus at an MOI of 10 in the presence of 1 μg/ml of anti-mIL10 antibody in culture. IL-12 was determined by ELISA. ▨ DCs infected with CPV alone, ⁘ DCs infected with CPV followed by LPS, ▥ DCs stimulated with LPS alone, ▦ no treatment, and ▤ DCs stimulated with CPV in the presence of anti-IL10. Values of zero indicate that IL-12 levels were below detectable limits. Values represent the mean of three replicates each (with the exception of the anti-IL-10 studies which were repeated only once). An asterisk (*) indicates that the differences in TNF-α production in response to the LPS was significantly different than from no treatment controls, as determined by two way ANOVA and Dunnett's multiple range test. It is important to note that the IL-12 production by DCs infected with cowpox virus and then stimulated with LPS was not different than no treatment controls.

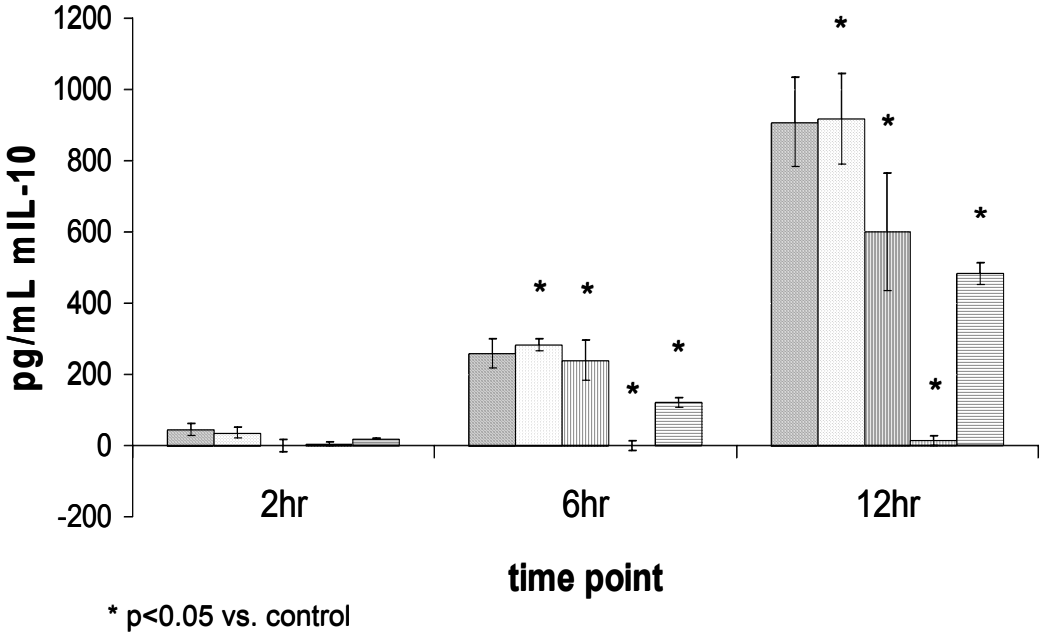

Figure 18. IL-10 production in DCs stimulated with LPS following a 6 hr. cowpox infection.

Note: DCs were infected with a recombinant cowpox virus expressing GFP at an MOI of 10 for two hours, and then allowed to rest for an additional six hours. The DCs were then stimulated with 10 μg LPS for two, six, and 12 hrs, as outlined in the Materials and Methods section. Additionally, some DCs were infected with cowpox virus at an MOI of 10 in the presence of 1 μg/ml of anti-mIL10 antibody in culture. IL-10 was determined by ELISA. DCs infected with CPV alone, DCs infected with CPV followed by LPS, DCs stimulated with LPS alone, no treatment, and DCs stimulated with CPV in the presence of anti-IL10. Values of zero indicate that IL-10 levels were below detectable limits. Values represent the mean of three replicates each (with the exception of the anti-IL-10 studies which were repeated only once). An asterisk (*) indicates that the differences in IL-10 production in response to the LPS was significantly different than from no treatment controls, as determined by two way ANOVA and Dunnett's multiple range test.

Because cowpox virus infection alone was shown to increase IL-10 production, DCs were also treated with an antibody against IL-10 during cowpox virus infection. IL-10 blockade appeared to increase both MHC class II and CD86 expression in response to CPV infection (Figures 19,20), although these studies were replicated only once.

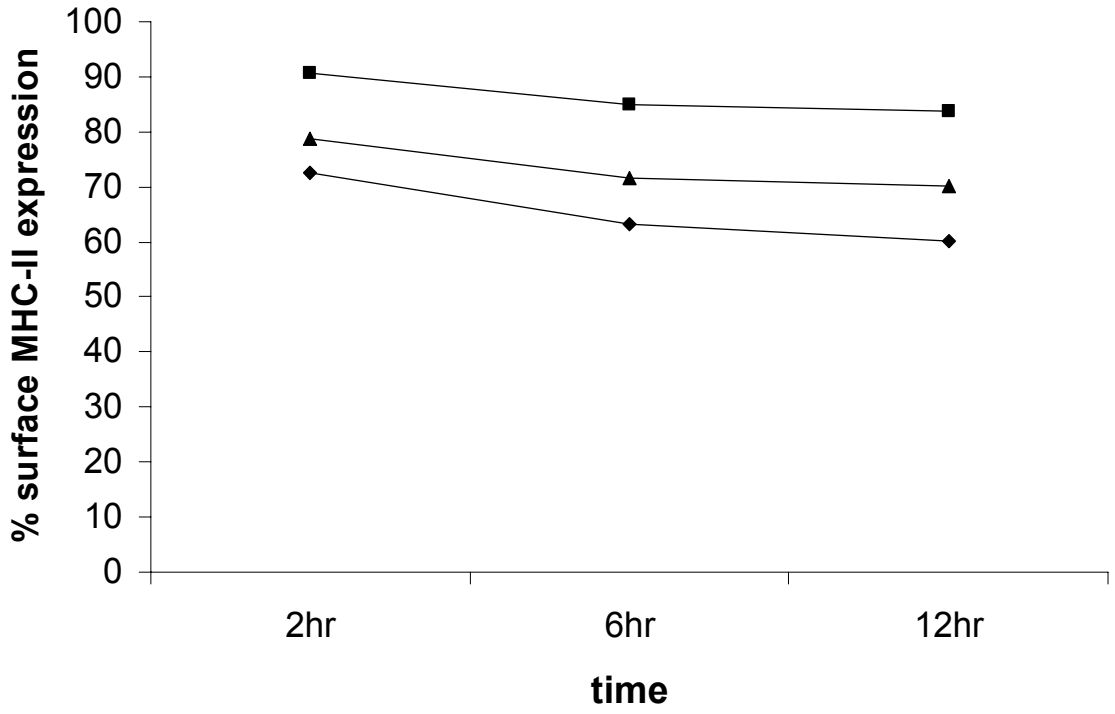

Figure 19. Flow cytometric analysis of MHC class II expression in DCs infected with cowpox virus in the presence of anti-IL10 in culture.

Note: DCs were infected with cowpox virus at an MOI of 10 in the presence or absence of anti-IL-10 antibody in culture for 2, 6, and 12 hrs. DCs were harvested and then stained for flow cytometry as outlined in the Materials and Methods section. GFP+ cells were recognized as cells infected with cowpox virus. Biotinylated antibodies to MHC-II were detected using APC and analyzed on a FACSCalibur® instrument. ♦----♦ no treatment controls, ■-----■ cowpox virus infection in the presence of anti-IL-10 antibodies, ▲-----▲ cowpox virus infection in standard culture medium. Values represent a single replicate. Although no statistics were performed because of the single replicate, there was a trend towards higher MHC class II expression in cells infected with cowpox virus in presence of neutralizing antibodies to IL-10.

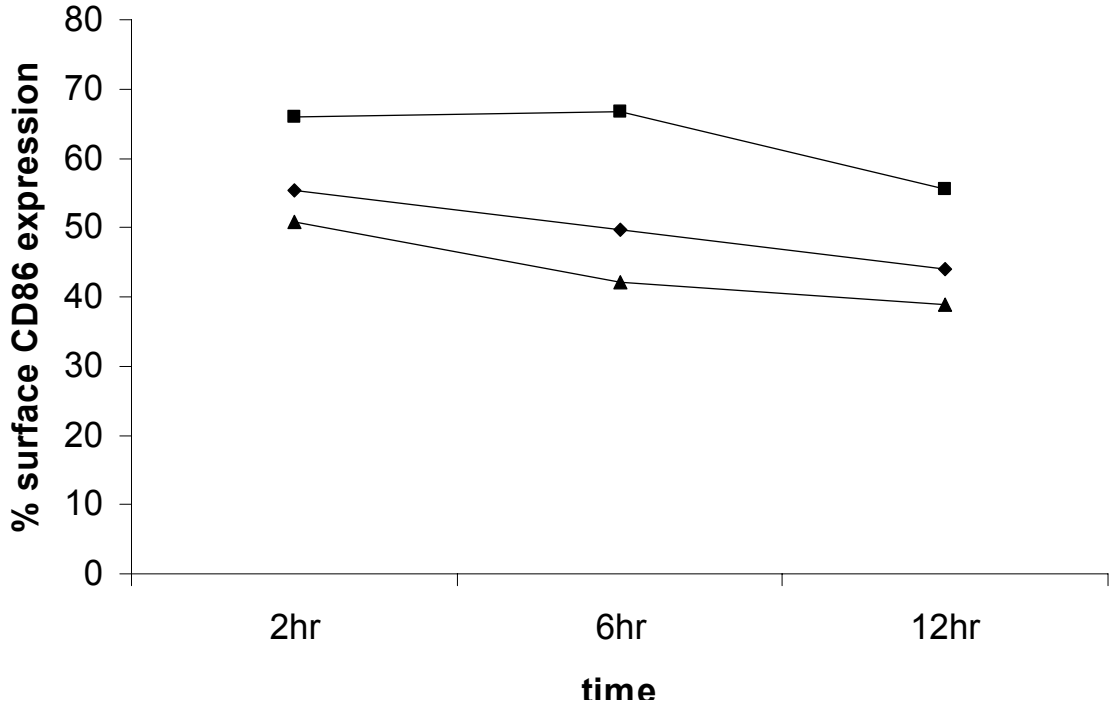

Figure 20. Flow cytometric analysis of CD86 expression in DCs infected with cowpox virus in the presence of anti-IL10 in culture.

Note: DCs were infected with cowpox virus at an MOI of 10 in the presence or absence of anti-IL-10 antibody in culture for 2, 6, and 12 hrs. DCs were harvested and then stained for flow cytometry as outlined in the Materials and Methods section. GFP+ cells were recognized as cells infected with cowpox virus. Biotinylated antibodies to CD86 were detected using APC and analyzed on a FACSCalibur® instrument. ♦----♦ no treatment controls, ■-----■ cowpox virus infection in the presence of anti-IL-10 antibodies, ▲-----▲ cowpox virus infection in standard culture medium. Values represent a single replicate. Although no statistics were performed because of the single replicate, there was a trend towards higher CD86 expression in cells infected with cowpox virus in presence of neutralizing antibodies to IL-10.

CHAPTER 4
DISCUSSION

The present studies examined the response of murine bone marrow derived DCs to ex vivo infection with cowpox virus. Cowpox virus is pathogenic in mice and has been used frequently in the past to study poxvirus pathogenesis (particularly pneumonia) in murine systems [36]. The DC maturation response to poxvirus was compared to the response to bacterial LPS, a prototypical inflammatory product of Gram negative bacteria. In addition, several additional studies were performed to examine the role of endogenous mediators, most notably TNF-α and IL-10 in modulating the DC response to poxvirus and to bacterial LPS.

The results were unequivocal. Infection of murine bone-marrow derived DCs with a pathogenic strain of cowpox virus resulted in dose-dependent infection of cells that led to cell death. Cell death was not immediate, and at MOIs of 1 or 10 pfu/cell, reduced cell vitality was evident by six to 24 hours. The incidence of cell death was not complete by 24 hours, with only a 55% reduction in cell vitality at that time point. This onset and degree of DC killing by cowpox virus is comparable to the degree of human peripheral blood monocyte derived DC killing by vaccinia, as reported by others [28,29]. Despite this significant biological effect, there was no evidence that infection with cowpox virus induced maturation of the DCs. Activation and maturation of DCs is a staged process that involves not only the increased presentation of cell surface proteins involved in antigen presentation (such as MHC class II, CD80, CD83 and CD86, as well as a variety of chemokine receptors), but also the synthesis and release of a number of cytokines,

including TNF-α, IL-12, IFN-α,β and IL-10, which are involved in the polarization of T cells. Bacterial lipopolysaccharide is a prototypical microbial pathogen recognized by TLR4 signaling, and is a potent activator of DC maturation [37]. A number of groups have shown that it is perhaps the most potent DC maturation agent (along with CD40 ligand), resulting in increased expression of MHC class II and CD86, and stimulates the release of TNF-α, IL-12, IFN-α,β and IL-10 [38]. The studies reported here demonstrate that the maturation of DCs in response to bacterial LPS is only modestly dependent upon TNF-α signaling. Although TNF-α is a strong maturation agent for DCs [39,40], the present studies suggest that maturation to LPS proceeds in a near normal fashion even in TNFR I null animals. CD86 and MHC class II expression were unaffected by LPS induced maturation in DCs from TNFR I null mice, although the production of TNF-α and IL-12p70 were modestly reduced. In contrast, it appears that an endogenous IL-10 response plays some role in moderating the DC maturation response to LPS. DCs obtained from IL-10 null mice showed an exaggerated increase in expression of CD86 and MHC class II, and a dramatic increase in the production of cytokines, IL-12p70 and TNF-α. Endogenous IL-10 production is presumed to suppress the production of Th1 cytokines and reduce the magnitude of the inflammatory response from systemic endotoxicosis [41].

In contrast to bacterial LPS, these studies demonstrated that cowpox virus infection failed to significantly induce the increased expression of either MHC class II or CD86 in these murine, myeloid derived DCs. In this regard, the studies are comparable to the work of Drillen et al. and Engelmayer et al., who examined human peripheral blood monocyte-derived DC maturation in response to *ex vivo* vaccinia virus infection [28,29].

In both those studies, the authors demonstrated that vaccinia virus infection was modestly lethal to the human DCs within 72 hrs, and failed to alter either the expression of MHC class II, CD83 or CD86 expression, or stimulate the production of TNF-α or IL-6. Interestingly, Drillen et al. [28] showed that a modified vaccinia virus Ankara, unlike wildtype vaccinia, induced a dramatic immunogenic response, and was capable of moderately activating human DCs. In our studies, the effect of cowpox virus infection on DC maturation was not simply an absence of any response. Rather, a significant release of IL-10 was observed in DCs infected with cowpox virus. This was not seen to the same degree with LPS stimulation. Interestingly, although these studies were performed only once, blocking the IL-10 response with a monoclonal antibody increased CD86 and MHC class II expression on the cowpox virus infected DCs. IL-10 is an important regulatory molecule produced by DCs that is known to have a number of biological effects on both antigen presenting cells and T lymphocytes. IL-10 is a potent suppressor of DC maturation and suppresses Th1 responses [42,43]. It is possible that the poxvirus production of IL-10 could have acted in an autocrine fashion to reduce cell surface expression. Interestingly, coincubation of immature DCs expressing IL-10 with naïve T cells is known to generate a population of T regulatory cells with potent immune suppressive properties [44,45]. These T regulatory cells are primarily responsible for suppressing antigenic responses to self. It is interesting to speculate that the failure of DCs to undergo a classical maturation and presentation of antigens to T cells may be one mechanism by which poxvirus can subvert the development of efficient antiviral immunity. Furthermore, the increased expression of IL-10 by these phenotypically immature DCs may lead to the expansion of the toleragenic T cells. Pathogenic poxvirus

in mice, such as cowpox virus, may employ these mechanisms as a tool for immune evasion.

More interesting is the observation that poxvirus infection suppressed the ability of these predominantly immature, myeloid derived DCs to undergo maturation to a subsequent prototypical maturation agent, bacterial LPS. Six hours of cowpox virus infection dramatically reduced the response of these immature myeloid DCs to a subsequent maturation signal by bacterial LPS. The increase in MHC class II expression was not affected by the prior cowpox virus infection, whereas CD86 expression was significantly reduced. This is not surprising since MHC class II molecules exist preformed in DCs and their presentation does not require new protein synthesis [46]. In addition, the expression of TNF-α and IL-12 were significantly reduced by the prior cowpox virus infection, whereas IL-10 expression was increased when compared to LPS treatment alone. Although these and other studies have demonstrated that cowpox virus infection can kill a significant proportion of DCs at 24 hours, and suppress the synthetic rate of endogenous proteins [47], the suppression in gene expression and protein synthesis are not necessarily complete. In this regard, the current studies extend the earlier findings of Drillen and Engelmeyer. Drillen et al. have argued that poxvirus gene expression is required to suppress DC activation [28], Engelmeyer et al. showed that immature human peripheral blood mononuclear cell DCs infected with vaccinia virus were also resistant to maturation by a cocktail of endogenous cytokines (IFN-α, TNF-α, IL-1β) [29].

Our findings would suggest that cowpox virus infections are more insidious than merely blocking the maturation response of DCs. We would conclude that the effect of

cowpox virus is not a passive effect on the innate immune response, but rather is an active process to generate a DC population that actively suppresses innate and acquired immune responses through the increased expression of IL-10.

As previously stated, DCs infected with cowpox virus are resistant to maturation and express high levels of IL-10. In addition, we can now show that DCs not directly infected by cowpox virus but in co-culture with infected cells also fail to show increased CD86 expression in response to LPS stimulation. This response was detected by using a cowpox virus recombinant that expresses GFP off a CMV promoter. DCs that express GFP are infected whereas DCs that are not expressing GFP were likely not infected by the virus. Under conditions of an MOI of 10, approximately 46% of the DCs were expressing GFP, consistent with their infection. GFP negative $CD11c^{+}$ cells infected with poxvirus also did not increase their expression of CD86 in response to LPS stimulation, suggesting that these cells were also resistant to activation by LPS. One possible explanation is that those DCs infected with cowpox virus (GFP expressing) are secreting a humoral factor that suppresses CD86 expression in the DCs that are not infected with cowpox virus.

Conclusions

The present findings suggest that cowpox virus, a pathogenic agent in the mouse, can readily infect myeloid-derived dendritic cells under *ex vivo* conditions. This infection is cytopathic to the cells but does not induce a classical DC activation, similar to that seen with bacterial lipopolysaccharide. Rather, the infected DCs secreted high levels of the anti-inflammatory cytokine, IL-10. In response to a subsequent prototypical inflammatory challenge, bacterial LPS, prior cowpox virus infection blunts the activation and maturation response. It appears that poxvirus infections can subvert the innate

immune response by DCs through a directed effort to prevent and modify the recognition by DCs. In addition, the DC response to poxvirus infection predisposes the host to a toleragenic phenotype through the increased expression of IL-10.

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BIOGRAPHICAL SKETCH

Justin DeBernardis was born on July 27th, 1979, in Altoona, Pennsylvania. He grew up there with his parents, Ross and Michele, younger brother Marc, and older sister Lori. In 1991, Justin and his family moved to Jacksonville, Florida. Justin graduated from Nease High School in 1997 and immediately enrolled at the University of Florida. He graduated with honors from the University of Florida with his Bachelor of Science in microbiology and cell science in 2001. Justin began his master's in medical sciences program with a concentration in immunology at the University of Florida in 2002. In 2003, he enrolled in a concurrent degree program to obtain his master's in business management, and finally graduated from both programs in the summer of 2004. Following graduation, Justin plans to pursue a project management career in the bio-pharmaceutical industry.